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Deletion of the Coffin–Lowry Syndrome Gene Rsk2 in Mice is Associated With Impaired Spatial Learning and Reduced Control of Exploratory Behavior

Poirier, R ; Jacquot, S ; Vaillend, C ; Souththiphong, A A ; Libbey, M ; Davis, S ; Laroche, S ; Hanauer, A ; Welzl, H ; Lipp, H-P ; Wolfer, D P

Abstract: Coffin-Lowry Syndrome (CLS) is an X-linked syndromic form of mental retardation associated with skeletal abnormalities. It is caused by mutations of the Rsk2 gene, which encodes a growth factor regulated kinase. Gene deletion studies in mice have shown an essential role for the Rsk2 gene in osteoblast differentiation and function, establishing a causal link between Rsk2 deficiency and skeletal abnormalities of CLS. Although analyses in mice have revealed prominent expression of Rsk2 in brain structures that are essential for learning and memory, evidence at the behavioral level for an involvement of Rsk2 in cognitive function is still lacking. Here, we have examined Rsk2-deficient mice in two extensive batteries of behavioral tests, which were conducted independently in two laboratories in Zurich (Switzerland) and Orsay (France). Despite the known reduction of bone mass, all parameters of motor function were normal, confirming the suitability of Rsk2-deficient mice for behavioral testing. Rsk2-deficient mice showed a mild impairment of spatial working memory, delayed acquisition of a spatial reference memory task and long-term spatial memory deficits. In contrast, associative and recognition memory, as well as the habituation of exploratory activity were normal. Our studies also revealed mild signs of disinhibition in exploratory activity, as well as a difficulty to adapt to new test environments, which likely contributed to the learning impairments displayed by Rsk2-deficient mice. The observed behavioral changes are in line with observations made in other mouse models of human mental retardation and support a role of Rsk2 in cognitive functions

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Deletion of the Coffin–Lowry Syndrome Gene *Rsk2* in Mice is Associated With Impaired Spatial Learning and Reduced Control of Exploratory Behavior

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Abstract Coffin–Lowry Syndrome (CLS) is an X-linked syndromic form of mental retardation associated with skeletal abnormalities. It is caused by mutations of the *Rsk2* gene, which encodes a growth factor regulated kinase. Gene deletion studies in mice have shown an essential role for the *Rsk2* gene in osteoblast differentiation and function, establishing a causal link between *Rsk2* deficiency and skeletal abnormalities of CLS. Although analyses in mice have revealed prominent expression of *Rsk2* in brain structures that are essential for learning and memory, evidence at the behavioral level for an involvement of *Rsk2* in cognitive function is still lacking. Here, we have examined *Rsk2*-deficient mice in two extensive batteries of behavioral tests, which were conducted independently in two laboratories in Zurich (Switzerland) and Orsay (France). Despite the known reduction of bone mass,

all parameters of motor function were normal, confirming the suitability of *Rsk2*-deficient mice for behavioral testing. *Rsk2*-deficient mice showed a mild impairment of spatial working memory, delayed acquisition of a spatial reference memory task and long-term spatial memory deficits. In contrast, associative and recognition memory, as well as the habituation of exploratory activity were normal. Our studies also revealed mild signs of disinhibition in exploratory activity, as well as a difficulty to adapt to new test environments, which likely contributed to the learning impairments displayed by *Rsk2*-deficient mice. The observed behavioral changes are in line with observations made in other mouse models of human mental retardation and support a role of *Rsk2* in cognitive functions.

Keywords Mental retardation · Coffin–Lowry Syndrome · Mouse model · Cognition · Learning and memory · Exploratory behavior · Ras/MAPK signaling

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Introduction

Mental retardation is a common human disorder characterized by impairment of intellectual functions and adaptive behaviors as expressed in conceptual, social, and practical skills. The disability is detected at birth or early in childhood, and may result from genetic alteration, environmental insult, or a combination of both. Whereas mental disability is the only symptom of the disease in non-syndromic forms, syndromic forms of mental retardation are characterized by a combination of mental disabilities with physical malformations and/or metabolic disturbances. Coffin–Lowry Syndrome (CLS) is a rare syndromic form of mental

retardation that shows X-linked inheritance with an estimated incidence of 1 in 50,000–100,000 males (Coffin et al. 1966; Lowry et al. 1971). It is characterized by moderate to severe psychomotor retardation, growth retardation (short stature), facial and digital dysmorphisms, as well as progressive skeletal deformations (Hanauer and Young 2002). Genetic analysis mapped the CLS locus to an interval of 2–3 megabases at Xp22.2 (Hanauer et al. 1988) and identified within that interval the ribosomal S6 kinase Rsk2 gene as the cause of CLS (Trivier et al. 1996). In humans, Rsk2 belongs to a family of four highly homologous proteins (RSK1–4), encoded by distinct genes.

The Rsk2 gene is subject to strong allelic heterogeneity. Over 130 mutations distributed throughout the gene have so far been identified in CLS patients, the majority being unique to single families with no obvious correlation with the severity of clinical features (Delaunoy et al. 2001; Jacquot et al. 1998; Touraine et al. 2002). Cognitive deficiencies in CLS patients are prominent, but markedly variable in severity, including between siblings (Hanauer and Young 2002). The vast majority of male patients are, however, severely affected. They suffer from a low intelligence quotient (IQ), ranging from 15 to 60, and retarded language skill acquisition that increases in severity with the occurrence of hearing loss in a few patients. Female carriers, however, fall along a continuum ranging from severe retardation to relatively normal intellectual performance. Although systematic assessment of learning and memory abilities in identified CLS patients with Rsk2 mutations is still lacking, learning and memory disabilities have been noted during examination of a few males and female carriers with relatively mild levels of mental retardation (R. Touraine and A. Hanauer, personal communication).

Rsk2 is a serine/threonine kinase and a cytosolic substrate in the Ras/MAPK signaling pathway. It is activated by extracellular signal-regulated kinase (ERK). Among the several cytosolic and nuclear substrates identified so far, Rsk2 can phosphorylate histones (Sassone-Corsi et al. 1999) and various transcription factors such as ATF4 and the cAMP response element binding protein, CREB (De Cesare et al. 1998; Frodin and Gammeltoft 1999; Yang et al. 2004), suggesting an important role for CRE-mediated transcription. Numerous studies implicate the MAPK/ERK signaling cascade and CREB-mediated gene transcription in synaptic plasticity and memory. For review, see Bozon et al. (2003), Davis and Laroche (2006) and Sweatt (2001, 2004). This suggests that loss of Rsk2 function may contribute to the cognitive deficits in CLS patients. Moreover, in brain, Rsk2 is expressed pre-

dominantly in cerebellum, hippocampus and neocortex (Zenou et al. 2002), regions that are implicated in various kinds of learning and forms of memory.

To study Rsk2 function *in vivo*, two lines of Rsk2-deficient mice were generated by targeted disruption of the Rsk2 homologue in mice (Dufresne et al. 2001; Yang et al. 2004). A first study in one line reported elevated and prolonged ERK activation in skeletal muscle of Rsk2-null mice, suggesting that Rsk2 plays a role in feedback inhibition of ERK in skeletal muscle (Dufresne et al. 2001). The same study also reported reduced body weight and length as well as motor coordination deficits associated with or resulting in impaired spatial navigation. Later, it was established that the body weight difference is largely caused by a specific loss of white adipose tissue (El-Haschimi et al. 2003). Rsk2-null mice also have impaired glucose tolerance as well as elevated fasting insulin and glucose levels. In a different line of Rsk2-null mice, bone formation was delayed during embryonic development and bone mass remained low throughout postnatal life (Yang et al. 2004). These skeletal abnormalities resembled those found in human CLS patients. The same study identified lack of phosphorylation of the Rsk2 substrate ATF4 in osteoblasts as a mechanism contributing to the skeletal phenotype. To date, the consequence of the gene's disruption for behavioral and cognitive abilities has not been explored systematically. Here, we report the results of experiments carried out independently in two laboratories in order to investigate the phenotype of Rsk2-deficient mice in several learning tasks including spatial working and reference memory, associative and recognition memory, as well as in behavioral tests for motor coordination and activity, exploratory behavior, and anxiety related responses.

Material and methods

Generation of animals

The generation of Rsk2-null mice by homologous recombination has been described previously (Yang et al. 2004). The targeting vector was constructed by inserting a neomycine resistance gene, flanked by two loxP sites and followed by three stop codons (in the three forward reading frames) in exon 2 of Rsk2. The construct was linearized and electroporated into 129X1/SvJ embryonic stem (ES) cells and NeoR clones were selected. The ES cells carrying the correct mutation were injected into C57BL/6J blastocysts. Resulting mixed-background 129X1/SvJ × C57BL/6J

mice carrying a targeted allele of *Rsk2* were backcrossed six times with C57BL/6J mice, before the NeoR cassette was removed by crossing with a C57BL/6J/CMV-Cre transgenic line. This produced mutant mice with a single LoxP followed by three stop codons within exon 2. Western blot analysis of various tissue protein extracts, including brain, showed absence of *Rsk2* protein in mutant mice. Females heterozygous for the mutation have been backcrossed for at least 15 generations to C57BL/6 males to generate *Rsk2*-null (KO) and littermate control mice (Wt) for the present study. The genotype of the mice was determined as described previously (Yang et al. 2004).

General procedures

Animal housing and testing

The behavior of *Rsk2*-deficient mice was investigated independently in two laboratories in Zurich (Switzerland) and Orsay (France). Upon arrival at the laboratories, mouse siblings were kept in groups (2–5 per cages) with food and water ad libitum. They were placed under a 12:12 h inverted cycle in Zurich (lights off 08:00–20:00), whereas they were maintained under standard illumination cycle in Orsay (lights on 07:00–19:00). Mice were placed in individual cages 1 week before all experiments in Zurich, and before the conditioned taste aversion and hole-board tasks in Orsay. Behavioral testing was undertaken between 08:00 and 20:00. A total of 202 male mice (107 Wt, 95 KO) distributed across eight cohorts were used. Five cohorts (79 Wt, 73 KO) were used in Zurich: Three cohorts were tested on a battery of tests in the following order: water-maze place navigation, open-field, elevated O-maze. A subset of these animals (39 Wt, 39 KO) was also tested on the radial maze. The fourth cohort was tested on a serial reversal procedure in the water-maze followed for some of the animals (9 Wt, 9 KO) by radial maze and emergence/object exploration tests. The fifth cohort was tested on the water-maze place navigation task, followed by an emergence/object exploration test. Three cohorts (28 Wt, 22 KO) were used in Orsay. Two of them were tested in a series of tasks in the following order: open-field activity and object recognition, water-maze place navigation, grid-suspension and traction reflex tests, spontaneous alternation in a cross maze, and conditioned taste aversion. The third cohort was tested on the elevated-plus maze followed by a hole-board task. In positively reinforced learning paradigms, a gradual food-restriction regimen was initiated 4 days before testing, and the mice were maintained at 80–85% of

their free-feeding weight throughout. All animals were tested between 2 and 7 months of age, and were tested in only one test per day (the exception was the grid and traction-reflex tests, which were conducted on the same day) with at least 48 h between distinct experiments. All experiments were conducted blind to the genotype. Behavioral procedures were approved by Swiss animal welfare authorities and conducted in accordance with the European Communities Council Directive of November 24, 1986, respectively.

Video tracking

Unless otherwise stated, animals tested in the water-maze and exploratory tests were video-tracked using ViewPoint 2.68 (Orsay) or Noldus EthoVision 3.0 (Zurich). These were used to record *xy* position, object area and status of defined event-recorder keys as required. Raw data recorded in Zurich were then transferred to the public domain software Wintrack 2.4 (www.dpwolfer.ch/wintrack) for further analysis (Wolfer et al. 2001).

Tests of motor coordination

Inverted grid test

Sensorimotor abilities and muscle strength were evaluated by placing each mouse in the middle of a wire grid inverted at a 180° angle. Time for the mice to remain upside down on the grid was counted, with a maximum of 60 s if the mouse did not fall.

Wire suspension test

To test both muscle strength and motor coordination, the forepaws of each mouse was placed on a thin horizontal wire (1.5 mm in diameter) 35 cm above a table surface. Latency to bring at least one hindpaw up to grip the wire was recorded. Each mouse was given three trials (10-min ITI) and the mean was calculated per mouse. Each trial lasted for a maximum of 25 s.

Exploration and emotional reactivity

Open field

The round open-field arena (diameter: 150 cm) had a white plastic floor, and sidewalls (35 cm high) made of white polypropylene. Illumination was by indirect diffuse room light (12 lux). Each mouse was released near the wall and observed for 10 min. The same procedure was repeated the following day. Three zones

were defined: a circular centre field (50% of arena surface), a transition zone and a wall zone (7 cm wide, 18% of arena surface). Video-tracked data were processed as described in detail previously (Drai and Golani 2001; Madani et al. 2003). Paths were segmented into three motion states: (i) bouts of progressive (long-distance) locomotion, (ii) periods of relative immobility (resting, grooming or freezing), (iii) episodes of small movements (lingering) which correlated with exploratory behaviors (brief stopping, sniffing, establishing snout contact with the apparatus or an object, looking around, stretching postures, rearing or leaning against the wall). Long distance locomotion was further characterized by calculating the median acceleration across all bouts of progressive locomotion (darting) (Kafkafi et al. 2003b). In order to assess path tortuosity and circling, the path of progressive movement was divided into straight segments and clockwise (negative) or counter-clockwise (positive) curves with a consistent direction change during 1 s or longer. The sum of all unsigned direction changes divided by total distance moved was then used as estimate of path tortuosity, while the sum of all signed direction changes divided by total distance moved served as circling index. To obtain an estimate of small scale gait stability, a wobbling index was calculated as the sum of unsigned direction changes between successive segments of the path, divided by the distance moved. As a measure of spatial and temporal scatter of exploration activity we calculated % of arena surface explored by dividing the arena into quadratic tiles of $5 \times 5 \text{ cm}^2$ and by counting tiles in which at least one scanning episode had occurred (Madani et al. 2003). Repeating sequences of tile visits were counted to obtain an index of large-scale stereotypical movements (Madani et al. 2003). Finally, diversity of exploration was estimated as average distance between any two scanning episodes, weighted by the distribution of their duration (Kafkafi et al. 2003a).

Elevated O-maze

The apparatus was a 5.5 cm wide annular runway (grey plastic, 46 cm outer diameter) placed inside the open-field arena 40 cm above the floor (Konig et al. 1996; Madani et al. 2003). The two opposing closed 90° sectors were protected by 16 cm high inner and outer walls. The remaining two open sectors had no walls. Animals were released in one of the closed sectors and observed for 10 min. The video-tracking system detected entries into open sectors only when the animal moved into it with all four paws. Head dips were recorded and classified as protected dips (occur-

ring when the animal was in a transition zone with its body partially between the protection walls) or unprotected dips (occurring after the animal had entered an open sector).

Elevated plus-maze

The plus-maze (black-hard plastic, 65 cm above the floor, equipped with a video camera) had two facing arms enclosed with high walls ($20 \times 8 \times 25 \text{ cm}$), two open arms ($20 \times 8 \text{ cm}$) and a central area ($8 \times 8 \text{ cm}$) to form a plus sign. Illumination was 150 lux in open and 30 lux in enclosed arms. Each mouse was placed in the central area with the head facing an open arm and observed for 15 min. Number of entries and time spent in open or enclosed arms were recorded by blocks of 5 min.

Emergence test and object exploration

Frames of non-reflective aluminum (37 cm high) were used to partition the open-field into four $50 \times 50 \text{ cm}$ arenas, for concurrent observation of four mice. During the emergence test, each arena had a $12 \times 8 \times 4 \text{ cm}$ plastic home box with an aperture of $6 \times 2.5 \text{ cm}$, positioned in a corner at 5 cm from the nearest wall, with the aperture facing away from the wall. The novel object was a $12 \times 4 \text{ cm}$ semi-transparent 50 ml Falcon tube positioned vertically in the centre of the arena. For analysis, we defined a central circular object zone of 18 cm in diameter and square shaped corner zones of $5 \times 5 \text{ cm}$. The day prior to the emergence test, a clean home box was placed in the home cage of each mouse. The next day, mice and home boxes were introduced into the arenas and observed for 30 min. The object exploration test was run one day later. Each mouse was observed for 30 min in the empty, clean arena. Then, the novel object was introduced and observation continued for another 30 min. Behavioral measures were calculated from video-tracked data using the same principles as in the open field test. In addition, vertical activity (which may include rearing, leaning, or other movements that do not translate into locomotion) was estimated by counting reductions of tracked subject size (number of pixels above threshold assigned to the subject) while the animal was not engaging in progressive movement (Madani et al. 2003). Automatic observer-independent estimates of object exploration (Berger et al. 2006; Madani et al. 2003; Wolfer et al. 2004; Zorner et al. 2003) were obtained based on methods introduced by Dulawa et al. (1999) by measuring small movements and vertical activity that occurred while the animal was

inside the object zone. With an object zone diameter of 18 cm, the tracking system registered activity to this zone whenever the animal was at least touching the object with its nose. To reduce error introduced by occasional, object-unrelated small movements inside the object zone, the measures were corrected by subtraction of the values obtained in the absence of the object during the habituation period.

Learning and memory

Spontaneous alternation in a cross maze

The apparatus consisted of a transparent cross maze with four arms ($7.5 \times 33 \times 11$ cm) radiating at 90° from a central area (7.5×7.5 cm). The maze was placed in a well-lit room (70–80 lux) with several extra-maze cues. Mice were individually placed in the centre of the cross maze and allowed to freely explore the four arms for 10 min. The number of visits and the temporal order of visits to individual arms were recorded. The mean number of different arms visited within every set of four arm entries was calculated. In addition, all sequences of 2, 3 or 4 consecutive visits (S2, S3, or S4) were analyzed separately and the number of sequences in which all visited arms were different (sequences of alternated choices) was quantified. Chance levels are 75% for S2, 37.5% for S3 and 9.37% for S4. As an indicator of stereotyped response patterns that may interfere with performance, chaining responses (visiting 3 or 4 arms in clockwise or anti-clockwise order) were quantified.

Open field activity and object recognition

The test box, equipped with a video camera, consisted of a square open-field ($50 \times 50 \times 50$ cm) with black walls and a white floor covered with sawdust. Experiments were undertaken under homogeneous dim illumination (<50 lux). The objects were small wooden or plastic toys of different colors and shapes (3–6 cm diameter \times 3–6 cm high) or made out of Lego® pieces ($6 \times 4 \times 3.5$ cm). Three objects were placed near the corners at 15 cm from the walls. The objects and their spatial arrangement in the test box were chosen in a pseudorandom order and were counterbalanced between mice. The testing procedure started with a 4-day period of habituation consisting of two daily sessions of 10 min separated by a 5-h delay, as previously described (Vaillend et al. 2004). On day 1, littermate mice from a given cage were placed all at once in the empty open-field and allowed to move freely for 10 min. On days 2–3, mice were individually exposed to the open field and spontaneous locomotor

activity was recorded. On day 4, two identical plastic objects that were not subsequently used were placed in the box for 10 min. The object discrimination tasks started 48 h after habituation. Each mouse was successively tested on two versions of the object discrimination task (spatial versus non-spatial) with a 24-h delay between the two experiments (Bozon et al. 2002). Each experiment consisted of a single acquisition session (two trials of 10 min with a 10-min ITI) followed by a 5-min retention test 24 h later. In the non-spatial version, one of the three objects was replaced by a novel object during the retention test, whereas one of the objects was moved to a novel position in the spatial version. Object changes during the test phase were counterbalanced among individuals and genotypes. Behavioral parameters were recorded minute by minute during each test phase. During pretraining, the arena was divided into virtual square sectors (5×5 grid) to quantify locomotor activity, expressed as the number of sectors crossed (horizontal activity) and the number of rearings and leanings (vertical activity). During both acquisition and retention phases, the latency of the first contact with an object and the time spent in contact with it were recorded. Contact was defined as the mouse's snout or paws touching the object. Retention performance was expressed as $100\% \times \text{time spent exploring the novel or displaced object} / (\text{time spent exploring the novel or displaced object} + \text{average time spent exploring the two unchanged objects})$.

Conditioned taste aversion

Three days prior to testing, mice were placed on a water-restriction regime with access to water for 30 min/day, from two identical bottles placed in their home cages. The bottles were weighed to evaluate fluid consumption. On the conditioning day, mice had free access to a 15%-sucrose solution for 30 min, in two identical bottles. One hour later, mice were injected (i.p.) with either 0.9% saline, or lithium chloride (LiCl: 0.3 M, 2% body weight). Twenty-four hours later, mice were given a two-bottle choice test between the water and sucrose for 30 min. The relative position of the two bottles was counterbalanced between mice. Conditioned taste aversion was expressed as the percent sucrose solution consumed over total fluid intake.

Spatial working memory procedure on the radial maze

The apparatus consisted of eight arms (7×38 cm, grey poly-vinyl chloride) with clear Perspex sidewalls (5 cm high) extending from an octagonal central platform

(diameter 18.5 cm). The maze was placed 38 cm above the floor in a dimly lit room (12 lux) rich in salient extra-maze cues. Single 6 mg millet-grains (Demeter Bio Goldhirse, Steiner Mühle AG, Zollbrück, Switzerland) were placed as baits in small metal cups (diameter 3 cm, 1 cm deep) at the end of the arms, in such a way that the mouse could not see them without completely entering the arm. Before each test session, the mouse was placed in a reversed box on the central platform. Food-deprived mice (85% of free-feeding weight) began with two habituation sessions during which they learned to eat pellets distributed all over the maze. A 10 day-training period (1 trial/day) ensued where each arm was baited with a single pellet and time taken to eat all the pellets was recorded. Each trial was given a maximum of 10 min. Working memory errors (re-entries into previously visited arms) were recorded in addition to two types of procedural errors: arm omission and bait neglect (visits to the bait zone without bait collection). Aborted choices (exit without reaching the goal zone) were counted as well. As indicators of stereotyped response patterns that may interfere with spatial learning we recorded chaining responses (visiting three or more arms in ascending or descending order), choice angle repetitions (e.g. 1–2–3–4 or 6–4–2) and the frequency of the preferred choice angle.

Hole-board task

The apparatus consisted of an elevated square board (50 × 50 cm; 90 cm above the floor) placed in a well-lit room (500 lux) with several extra-maze cues. A food cup (2.5 cm wide/2 cm deep) was placed below the surface of the floor of the board, 14 cm from each corner. Beneath each of the four food cups, there were five visible but inaccessible pellets to prevent the use of olfactory cues. Mice were first habituated to the task for 3 days (one 10-min session per day; four pellets were placed in each cup and 16 scattered on the board on day 1, then only four pellets in the cups on days 2–3). Following habituation mice were tested for 5 days in a spatial discrimination task, with only one food cup baited with one pellet. Testing continued until mice reached a criterion of choosing the correct hole first on eight out of nine consecutive trials or until 60 trials had been given (Brosnan-Watters and Wozniak 1997). On each trial, mice were placed on the board until the pellet was retrieved and consumed. Between trials, mice were returned to their home cages and the hole-board was randomly rotated and cleaned with alcohol, to prevent the use of olfactory cues. The number of trials required to reach criterion was recorded.

Spatial learning in the water-maze was assessed using four different place navigation procedures. A procedure with two blocks of four trials per day for 11 days was used in Orsay. Six trials × 5 days, 2 trials × 14 days and a serial reversal procedures were performed in Zurich.

Water-maze two blocks of four trials per day for 11 days

The maze consisted of a circular tank (150 cm diameter) filled with water (22°C) to 15 cm below the top of the sidewall, made opaque by addition of a white non-toxic paint (Opacifier 631, Morton SA, France). A circular escape platform (10-cm diameter) was placed in the centre of the maze during pre-training or the centre of a quadrant (35 cm from the wall) during training. The platform, placed 0.5 cm below the water surface, was not visible. The maze was placed in a well-lit room (380 lux) containing several extra-maze cues on the walls. A video camera, mounted on the ceiling above the maze to record swim paths, was connected to a computer located in an adjacent room. The day before training, mice underwent 2 pre-training sessions (four trials in the morning and afternoon). An habituation session started with the mouse standing on the platform for 60 s in the centre of the maze. Then, a trial started by introducing the mouse into the maze facing the wall at one of the four designated starting points in a pseudorandom order. Immediately, the mouse was gently guided by hand to the platform and allowed to remain on it for 60 s. During the training phase, mice were given two blocks of four trials a day for 11 days. The two trial blocks were separated by a 5-h interval. During each block, the mouse was introduced into the maze from three different starting points and allowed to swim freely until it reached the platform. Mice failing to find the platform after 90 s were gently guided to it by hand and a maximum escape latency of 90 s was recorded. Mice were allowed to remain 60 s on the platform before the start of the next trial. Probe tests were performed 24 h and 9 days after the last training session in both groups of mice. They consisted of a single trial during which the platform was removed and mice were allowed to search the platform for 90 s. After the first probe test (24 h), mice were given four additional training trials to prevent extinction. The data, recorded by video-tracking were used to reconstruct swim paths and to calculate averaged swim speed, swim path lengths and time spent in various virtual areas of the maze: the four quadrants; the four platform annuli, four extended annuli of 48-cm in diameter beyond that of the

platform, and a virtual corridor 19 cm in width, set along the wall to quantify thigmotaxis. Memory retention was evaluated during the probe test by comparing the time spent in the quadrant which previously contained the platform, to chance level (25%) and the annulus crossing index which represents the number of crosses over the platform site in the quadrant that contained the platform divided by crosses over identical areas in equivalent positions in target, adjacent left, adjacent right, and opposite quadrant.

Water-maze 6 trials \times 5 days

A round white propylene swim tank (150 cm diameter) was placed under dim light (12 lux) and filled with water (temperature 24–26°C) made opaque by addition of milk (Mohajeri et al. 2004). A white escape platform (14 \times 14 cm) made of wire mesh was submerged 0.5 cm below the water surface and remained in the same position during all training trials of the same animal. To minimize handling, mice were transferred to the pool using a white plastic cup and released a pseudo-randomly distributed points along the periphery of the pool. After they had reached the platform and stayed on it for 5 s, they were allowed to climb onto a wire mesh grid and transferred without further handling to their cage under a red warming lamp. The mice were trained for 5 days with six trials (max 120 s) per day (45 min average ITI). During the first 3 days (acquisition phase), the platform remained in a constant position and was then moved to the opposite quadrant for the remaining 2 days (reversal phase). The first 60 s of the first trial of the reversal phase served as probe trial to test for spatial retention. Learning was assessed by escape latency, swim path length, time spent floating (episodes of immobility or decelerations with speed minimum <0.06 m/s), and swim speed (excluding floating episodes). Path efficiency was defined as percent path during which the speed vector component pointing toward the goal was 75% or more. Wall oriented behavior was quantified by determination of percent time spent in a 10-cm wide wall zone, number of wall contacts, and percent path traveled parallel to the pool wall (path not meeting the efficiency criterion and having a radial speed vector component of 25% or less). Further, we calculated a circling index (absolute value of sum of all signed direction changes divided by distance swum). Spatial retention during the probe trial was assessed using percent time in quadrant and number of annulus crossings (target versus non-target quadrants). The annulus crossing index was calculated as specified above.

Water-maze 2 trials \times 14 days

This experiment used the same apparatus and general procedures as described above. Animals were trained for 14 days with two trials (max 90 s) per day and an ITI of 60 s. Two 60-s probe trials were given, one on day 11 before the two training trials, the second 24 h after the last training day.

Water-maze serial reversal

The experiment used the same apparatus and general procedures as described above. The mice were pre-trained in a cue navigation task for 2 days with six trials each. During this phase, the platform was marked with a flag and moved to a new position for every trial. Then, mice had to learn five consecutive positions of a hidden platform as previously described (Chen et al. 2000). The maximum number of training days was 5 for the first platform position and 4 for the following platform positions. Mice were trained for a maximum of eight trials per day, with a 10-min ITI during which they were returned to their cages. The mice were trained to the same platform position until they reached the criterion of three consecutive trials with an escape latency ≤ 15 s, or until completing the maximum number of training trials for each platform position. When the criterion was reached, the training was stopped and the mouse was then tested on a new platform position the following day.

Statistical analysis

Data were analyzed using ANOVA with genotype (Wt, KO) as the between-subject factor. Where more than one cohort had been tested, cohort was introduced as an additional between-subject factor to reduce unexplained variance and to determine whether genotype effects were cohort dependent. Because this was not the case, the cohort factor is not shown in the results. Similarly, we included absence/presence of the CMV-Cre transgene as additional between-subject factor in the analysis of the Zurich open-field, O-maze, radial maze, and 6 trials \times 5 days water-maze data because part of the animals still carried the CMV-Cre transgene that had served to remove the NeoR cassette. As there were no effects or interactions associated with this factor, it is not reported in the tables and figures. When required, the models were complemented by a within-subject factor to examine time or place dependence of effects. Statistical computations were done using Statview 5.0 (www.statview.com, no longer sold). Partial omega scores

(the proportion of variance accounted for by genotype if only this factor were in the design, range 0–1) were calculated manually using the ANOVA tables provided by Statview (Keren and Lewis 1979). An alpha value of $p < 0.05$ was considered statistically significant. In order to correct for multiple comparisons, conceptually related variables were grouped and significance thresholds adjusted using the false discovery rate control procedure by Benjamini et al. (Benjamini et al. 2001; Benjamini and Hochberg 1995).

Results

Rsk2-deficient mice appeared healthy and showed no overtly abnormal spontaneous behavior. They were 0.5–0.7 g lighter than controls before and after the 6 trials \times 5 days water-maze procedure, but showed similar weight loss during the experiment (Zurich: 45 KO, 25.5 ± 3.3 g before, 24.4 ± 3.1 g after; 52 Wt, 26.2 ± 3.2 g before, 24.9 ± 3.2 g after; genotype $F(1,85) = 6.8$, $p < 0.0108$, time $F(1,85) = 196.2$, $p < 0.0001$, genotype \times time $F(1,85) = 2.3$ ns). Body weights measured before the holeboard task showed no significant effect of genotype, but again Rsk2-deficient mice were on average 0.6 g lighter (Orsay: 11 KO, 28.9 ± 0.3 g and Wt, $n = 13$, 29.5 ± 0.6 g; $F(1,22) = 0.605$, $p < 0.4450$). There was no genotype effect on video-tracked subject size during the emergence and object exploration tests (Table 1). Rsk2-deficient mice were not impaired in sensorimotor tests involving muscle strength and motor coordination. In the inverted grid test, the mean time to remain suspended to the grid was similar in control (60 ± 0 s; $n = 15$) and Rsk2-deficient mice (56.09 ± 3.34 s; $n = 11$) mice (genotype $F(1,24) = 1.78$ ns). In the traction reflex test, there was no significant genotype effect on the mean latency to bring hind-paws up to the wire, but Rsk2-deficient mice had on average slightly longer latencies (6.03 ± 1.17 s; $n = 11$) as compared to controls (3.91 ± 0.62 s; $n = 15$) across the three consecutive trials (genotype $F(1,24) = 2.96$, $p = 0.0982$).

Exploration and emotional reactivity

In the *open-field* as well as while exploring the empty arena in preparation of the object recognition task, Rsk2-deficient mice showed normal levels of locomotor activity over several days of testing (Fig. 1A, Table 1). Habituation of locomotor activity across two days in the open field test was similar to controls (Fig. 1A). Mutant and control mice spent equal amounts of time with progressive locomotion, small

exploratory movements and resting. Moreover, irrespective of genotype, the mice showed a strong avoidance of the central zone of the open-field arena (Fig. 1B, Table 1). Fine-level analysis of locomotor patterns expressed in the large open-field revealed no evidence for neurological deficits or gait instability: indices of circling, path tortuosity, and wobbling were unaffected by the mutation. Also, exploratory excursions of Rsk2 mice were as spatially diverse as in control mice, and there was no evidence for abnormal large-scale stereotypical movements (Table 1). However, we found a clear increase in acceleration during episodes of progressive movement in Rsk2-deficient mice, indicating increased darting behavior (Kafafi et al. 2003b).

On the elevated *O-maze*, Rsk2-deficient mice and controls were similar with respect to general activity and avoidance of open sectors (Fig. 1C, Table 1). Exploratory head dips were overall slightly more frequent in mutants, but the ratio of protected to unprotected dips was indistinguishable from controls. In the *plus-maze* test, mice showed genotype-independent avoidance of the open arms as well (Fig. 1D). When exposed to a novel arena that offered a retreat opportunity in the form of a small familiar home box (*emergence test*), Rsk2-deficient mice were more active than wild-type mice but showed a similar rate of habituation (Fig. 1E, Table 1). They spent clearly less time in the home box, and showed reduced avoidance of the central zone of the arena. When a *novel object* was introduced into the now familiar arena following a 30-min period of habituation, Rsk2-deficient mice spent less time retreating to the corners, displayed an increased tendency to engage in large-scale stereotypical movements, and had elevated scores of estimated vertical activity. There was no significant genotype effect on measures estimating object-directed exploratory activity, but Rsk2-deficient mice were on average slightly faster to approach the object for the first time (Fig. 1G,H, Table 1). Significantly increased exploration of novel objects was detected in Rsk2-deficient mice during the acquisition phase of the object recognition task. When encountering objects for the first time during the first training phase, Rsk2-deficient mice showed increased object investigation, whereas exploration times were comparable between genotypes when mice were exposed to objects a second time 48 h later (Fig. 1F).

Learning and memory

To examine spatial working memory, we used an 8-arm radial maze and the spontaneous alternation paradigm

Table 1 Factorial ANOVA of exploration and anxiety tests

	<i>F</i> (df1,df2)	Type-I <i>p</i>	ω^2_P	FDR
<i>Open field (Zurich: 45 KO, 53 Wt)</i>				
Total distance moved	<i>F</i> (1,88) = 0.2	ns		
Progression velocity	<i>F</i> (1,88) = 2.4	↑ns		
% Time progressing	<i>F</i> (1,88) = 0.3	ns		
% Time immobile	<i>F</i> (1,88) = 0.4	ns		
Median acceleration (darting)	<i>F</i> (1,88) = 13.7	↑ <i>p</i> < 0.0004	0.113	Sig.
Circling index	<i>F</i> (1,88) = 1.6	↓ ns		
Tortuosity index	<i>F</i> (1,88) = 0.1	ns		
Wobbling index	<i>F</i> (1,88) = 0.1	ns		
Distance to center	<i>F</i> (1,88) = 0.1	ns		
% Arena explored	<i>F</i> (1,88) = 1.2	ns		
Diversity of exploration	<i>F</i> (1,88) = 0.3	ns		
Large-scale stereotypy of locomotion	<i>F</i> (1,88) = 0.2	ns		
<i>Open field (Orsay: 11 KO, 15 Wt)</i>				
Sectors crossed total	<i>F</i> (1,24) = 0.9	ns		
Rearings total	<i>F</i> (1,24) = 1.3	ns		
Sectors crossed in center	<i>F</i> (1,24) = 2.4	↓ns		
Rearings in center	<i>F</i> (1,24) = 1.2	ns		
Time in center	<i>F</i> (1,24) = 0.1	ns		
<i>O-maze test (Zurich: 47 KO, 53 Wt)</i>				
Total distance moved	<i>F</i> (1,87) = 1.9	↑ns		
Head dips	<i>F</i> (1,87) = 6.6	↑ <i>p</i> < 0.0117	0.054	Sig.
% Protected head dips	<i>F</i> (1,87) = 0.9	ns		
<i>Emergence test (Zurich: 19 KO, 21 Wt)</i>				
Tracked subject size	<i>F</i> (1,36) = 1.0	ns		
Total distance moved	<i>F</i> (1,36) = 5.4	↑ <i>p</i> < 0.0260	0.099	Sig.
Distance to center	<i>F</i> (1,36) = 8.4	↓ <i>p</i> < 0.0063	0.157	Sig.
Time in box	<i>F</i> (1,36) = 12.5	↓ <i>p</i> < 0.0011	0.225	Sig.
<i>Object exploration (Zurich: 19 KO, 21 Wt)</i>				
Tracked subject size	<i>F</i> (1,36) = 1.3	ns		
Total distance moved	<i>F</i> (1,36) = 1.9	↑ ns		
Estimated overall vertical activity	<i>F</i> (1,36) = 6.1	↑ <i>p</i> < 0.0185	0.113	Sig.
stereotypy count	<i>F</i> (1,36) = 12.1	↑ <i>p</i> < 0.0013	0.218	Sig.
% Time in corner	<i>F</i> (1,36) = 5.8	↓ <i>p</i> < 0.0218	0.106	Sig.
Latency to first object contact (log)	<i>F</i> (1,36) = 5.4	↓ <i>p</i> < 0.0262	0.099	
Horizontal object exploration	<i>F</i> (1,36) = 0.4	ns		
Vertical object exploration	<i>F</i> (1,36) = 0.4	ns		

Note: Type I error *p*-values are shown if <0.1, followed by estimated effect sizes as partial omega squared. Arrows indicate direction of mean differences if type I error *p* < 0.25. Conceptually related variables were grouped and significance levels adjusted using the false discovery ratio control (FDR) procedure. Effects considered statistically significant are flagged in the last column of the table

in a cross-maze. In the *8-arm radial maze* task, Rsk2-deficient mice showed a modest impairment (Fig. 2A, Table 2). The number of working memory errors was significantly increased with all training days contributing equally to the effect. The fact that mutants made more errors than controls only before taking the last bait, indicates that error probability increased with the amount of spatial information to be retained within a trial (Fig. 2B). Bait neglect and arm omission errors were as infrequent as in controls, indicating normal adaptation to the task situation. Mutants did not show an increased tendency for chaining responses or choice angle repetitions, but aborted arm visits occurred more often than in controls. *Alternation rates* in the cross-maze were significantly different from chance in both genotypes, but mutants also visited more arms in the available time than controls (Table 2). There was no significant effect of genotype on average number of different arm choices within every set of four arm

entries, but Rsk2-deficient mice obtained on average slightly poorer scores (Fig. 2C, Table 2).

Rsk2-deficient mice were not impaired during acquisition of the appetitive spatial learning task in the *hole-board*, which required the use of distal cues to locate a food pellet. On the contrary, mutant mice reached the learning criterion faster than controls on the first training day (Fig. 2D). Whereas scores reached on this first session may reflect differences in procedural, attentional or motivational factors, the learning curve established over the following training days suggests that spatial learning and consolidation processes were not impaired in Rsk2-deficient mice in this task. Also, no impairment was found in Rsk2-deficient mice during acquisition and retention (24 h delay) of the *object recognition* tasks, with both Rsk2-deficient and control mice showing an indistinguishable and robust preference for the novel and displaced objects 24 h after training (Fig. 2E). This suggests that

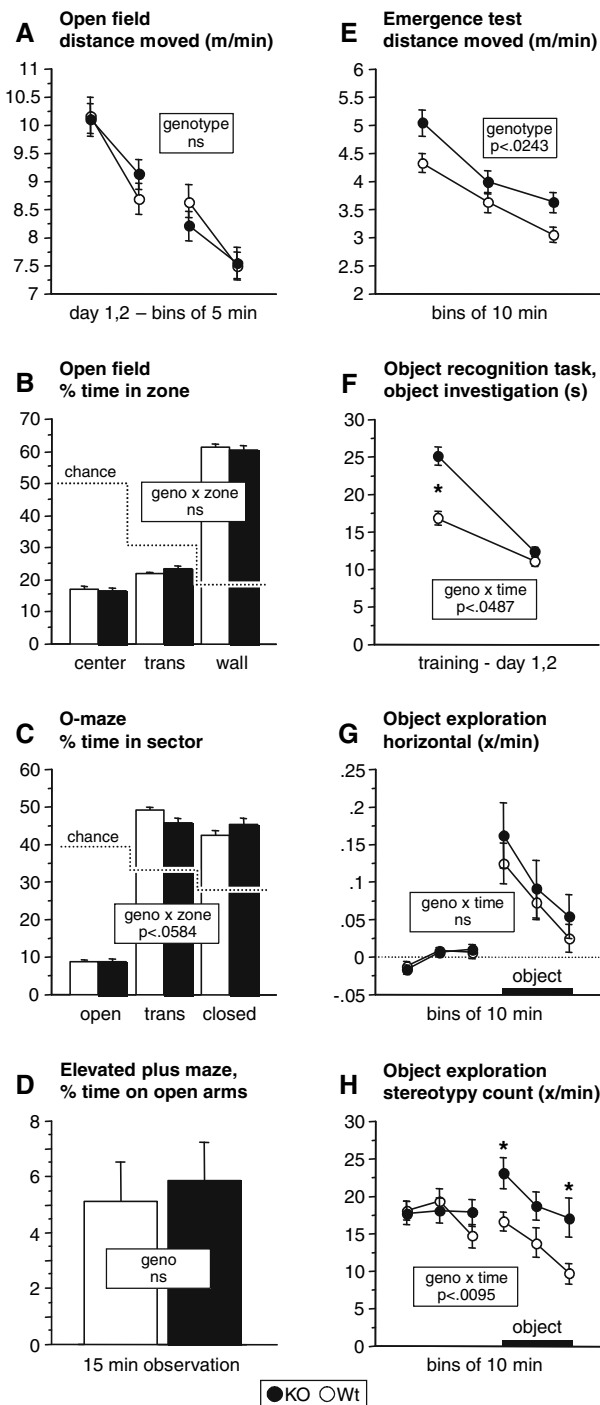
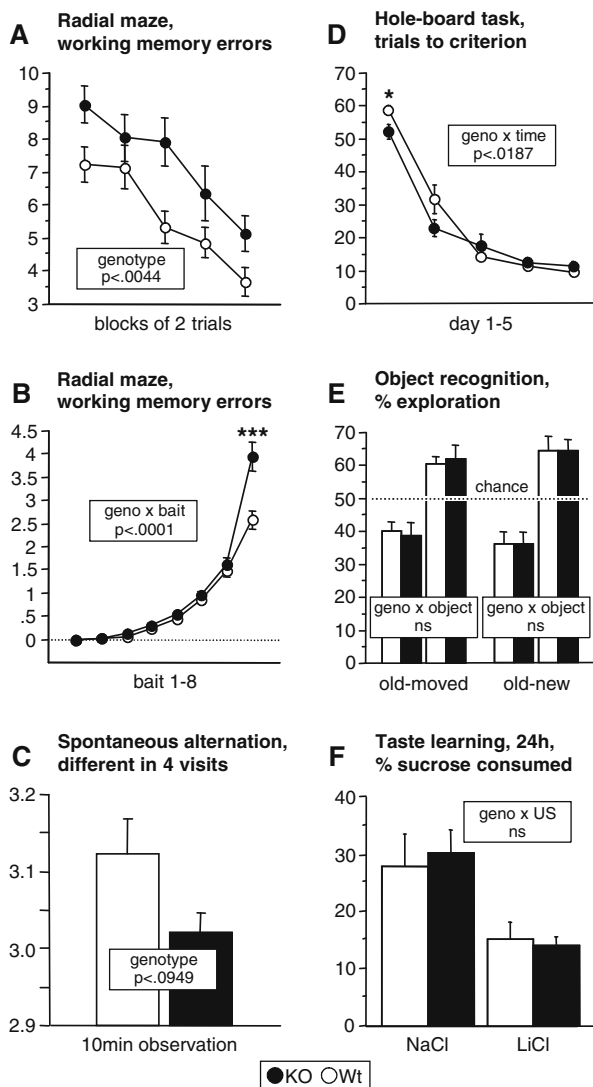


Fig. 1 Exploration and anxiety tests. **(A)** Distance moved in the open field during the first and second 5 min of the first and second day (Zurich: 47 KO, 53 Wt). Rsk2-deficient and control mice were indistinguishable with respect to total distance moved. Habituation of locomotor activity across two days in the open field test was similar to controls, even though Rsk2-deficient mice showed slightly more between day habituation and somewhat less within day habituation (genotype $F(1,86) = 0.1$ ns, time $F(3,258) = 73.6$, $p < 0.0001$, genotype \times time $F(3,258) = 2.8$, $p < 0.0382$, time in Wt $F(3,141) = 35.9$, $p < 0.0001$, time in KO $F(3,117) = 42.0$, $p < 0.0001$). **(B)** Time spent in the center, transition and wall zone of the open field. The stippled line indicates chance levels based on relative zone area. Both Rsk2-deficient mice and controls strongly avoided the center field in favor of the wall zone (zone $F(2,176) = 644.0$, $p < 0.0001$, genotype \times zone $F(2,176) = 0.4$ ns; time in center versus 50% chance: $t(99) = -60.192$, $p < 0.0001$). **(C)** Time spent on the open/closed sectors of the O-maze and in transition zone between the two (Zurich: 47 KO, 52 Wt). The stippled line indicates chance levels based on relative sector and zone size. Both groups strongly avoided the open sectors with Rsk2-deficient mice spending slightly more time in the transition zone (zone $F(2,174) = 549.6$, $p < 0.0001$, genotype \times zone $F(2,174) = 2.9$, $p < 0.0584$; genotype on open sectors $F(1,87) = 0.2$ ns, genotype in transition zone $F(1,87) = 4.7$, $p < 0.0323$, genotype on closed sectors $F(1,87) = 2.2$ ns; time on open sector versus 39% chance: Wt: $t(98) = -74.824$, $p < 0.0001$, KO $t(98) = -44.655$, $p < 0.0001$). **(D)** Percent time on the open arms of the elevated plus maze (Orsay: 11 KO, 13 Wt). The two groups showed the same strong avoidance of the open arms (genotype $F(1,22) < 0.1$ ns; time on open arms versus 50% chance: $t(23) = 45.353$, $p < 0.0001$). **(E)** Distance moved during the emergence test plotted in bins of 10 min (Zurich: 19 KO, 21 Wt). Rsk2-deficient mice were overall more active than controls but showed similar habituation during the 30 min observation period (genotype $F(1,36) = 5.5$, $p < 0.0243$, time $F(2,72) = 125.3$, $p < 0.0001$, genotype \times time $F(2,72) = 2.4$, $p < 0.0943$). **(F)** Object investigation time during the two acquisition trials of the object recognition test (Orsay: 11 KO, 15 Wt). On the first day, Rsk2-deficient mice spent more time investigating the objects than controls (genotype $F(1,24) = 3.8$, $p < 0.0622$, day $F(1,24) = 29.2$, $p < 0.0001$, genotype \times day $F(1,24) = 4.3$, $p < 0.0487$, genotype on day 1 $F(1,24) = 4.6$, $p < 0.0420$). **(G)** Time course of estimated horizontal exploratory activity directed toward the object during the object exploration test, plotted in bins of 10 min. The first three points show the habituation phase without object (Zurich: 19 KO, 21 Wt). Estimated object exploration was not significantly increased in Rsk2-deficient mice (genotype $F(1,36) = 0.4$ ns, time $F(5,180) = 20.4$, $p < 0.0001$, genotype \times time $F(5,180) = 0.4$ ns). **(H)** Large-scale stereotypical movements during the object exploration test, plotted in bins of 10 min. In presence of the object, Rsk2-deficient mice displayed more repetitive locomotion than controls (genotype $F(1,36) = 4.2$, $p < 0.0489$, time $F(5,180) = 5.5$, $p < 0.0001$, genotype \times time $F(5,180) = 3.2$, $p < 0.0095$; genotype during first, second and third 10 min of object exploration: $F(1,36) = 7.0$, $p < 0.0121$, $F(1,36) = 2.4$ ns, $F(1,36) = 7.0$, $p < 0.0121$).

long-term object recognition memory and long-term memory of the spatial arrangement of objects is not affected by the lack of Rsk2. Long-term associative memory was assessed in a conditioned *taste aversion* task. Compared with unconditioned controls, both Rsk2-deficient and control mice consumed significantly less of the sucrose solution 24 h after sucrose consumption had been paired with malaise induced by LiCl (Fig. 2F).

When tested in a *place navigation* task with two blocks of four trials per day and an ITI of 60 s, Rsk2-deficient mice showed a severe acquisition impairment, characterized by very slow learning (Fig. 3A, Table 3).



Mutant mice spent much more time near the wall. They took much more time and swam a much longer distance to reach the goal. Even though mutants swam more slowly, the massively increased swim path lengths indicate that the speed difference accounted only for a minor part of the performance deficit. Towards the end of training, Rsk2-mutant mice improved their performance and despite the dramatic delay in learning they were not significantly impaired in a probe trial given 24 h after the end of training (Fig. 3B, Table 3). When the probe trial was repeated 9 days later, there was a pronounced and statistically significant deficit (Fig. 3C, Table 3).

Another group of Rsk2-deficient mice were assessed in a protocol using 6 trials/day and an ITI of 45 min. Again, Rsk2-deficient mice had increased escape latencies and longer swim paths (Fig. 3D, Table 3), but the impairment was much smaller than in the

◀ **Fig. 2** Spatial learning in dry land mazes and associative memory. **(A)** Working memory errors on the 8-arm radial maze (Zurich: 48 KO, 48 Wt). Each point represents two trials. Rsk2-deficient mice made more errors than controls throughout training (genotype $F(1,88) = 8.6$, $p < 0.0044$, time $F(4,352) = 4.2$, $p < 0.0001$, genotype \times time $F(4,342) = 1.0$ ns). **(B)** Working memory errors by bait (errors before 1st bait, between 1st and 2nd, 2nd and 3rd, etc.). Rsk2-deficient mice made more working memory errors only while attempting to retrieve the very last bait (genotype $F(1,88) = 9.0$, $p < 0.0035$, bait $F(7,616) = 144.1$, $p < 0.0001$, genotype \times bait $F(7,616) = 4.8$, $p < 0.0001$; genotype during retrieval of last bait $F(1,88) = 13.2$, $p < 0.0005$). **(C)** The mean number of different arms visited within every set of four arm entries on the cross maze was not significantly reduced in Rsk2-deficient mice (Orsay: 11 KO, 15 Wt; genotype $F(1,24) = 3.0$, $p < 0.0949$). **(D)** Trials to criterion in the hole-board task (Orsay: 11 KO, 13 Wt). Points represent testing days. Rsk2-deficient mice required less trials to reach criterion on the first day and were indistinguishable from controls thereafter (genotype $F(1,22) = 1.2$ ns, time $F(4,88) = 145.1$, $p < 0.0001$, genotype \times time $F(4,88) = 3.1$, $p < 0.0187$; genotype day 1: $F(1,22) = 6.9$; $p = 0.0157$). **(E)** Percent exploration time of familiar versus changed object in the object discrimination task after a delay of 24 h (Orsay: 11 KO, 15 Wt). The first four bars show the results of the trial in which one of the objects had been displaced, the second four those of the trial in which one object had been replaced by a new one. In both tests the mice showed increased interest in the changed object regardless of their genotype (object displacement: object $F(1,24) = 24.6$, $p < 0.0001$, genotype \times object $F(1,24) = 0.1$ ns; object exchange: object $F(1,24) = 29.2$, $p < 0.0001$, genotype \times object $F(1,24) = 0.0$ ns). **(F)** Percent sucrose (sucrose/sucrose + plain water) consumed during the choice test 24 h after pairing of sucrose with either LiCl (5 KO, 7 Wt) as unconditioned stimulus or NaCl (5 KO, 8 Wt) as control (Orsay). Regardless of genotype the mice consumed less sucrose if it had previously been paired with LiCl induced nausea (genotype $F(1,21) < 0.1$ ns, US $F(1,21) = 10.1$, $p < 0.0045$, genotype \times US $F(1,21) = 0.1$ ns)

experiment shown in Fig. 3A. Mutants did not spend significantly more time near the wall, but approached the wall slightly more often. Their swim speed was indistinguishable from that of controls and there was no increased tendency for passive floating. Toward the end of training, performance of Rsk2-deficient mice was comparable to that of controls, and during the probe trial, 24 h later, they were normal as judged from % quadrant time and the annulus crossing index (Fig. 3E, Table 3). They did, however, spend more time in the wall zone of the pool. When the platform was moved to the opposite quadrant after 3 days of training, Rsk2-deficient mice had initially longer swim paths than controls but became as fast as controls within a few trials (Fig. 3D). Like controls, they abandoned searching the old goal quadrant after four reversal trials (Fig. 3F).

The acquisition deficit of Rsk2-deficient mice became stronger in the same pool when another cohort

Table 2 Factorial ANOVA of working memory tasks

	$F(df1,df2)$	Type-I p	ω^2_p	FDR
<i>8-arm radial maze (Zurich: 48 KO, 48 Wt)</i>				
Working memory errors	$F(1,99) = 7.6$	$\uparrow p < 0.0043$	0.073	Sig.
Correct before first working memory error	$F(1,99) = 2.4$	$\downarrow p < 0.0890$	0.020	
Correct choices in first 8	$F(1,99) = 1.4$	$\downarrow ns$		
Bait neglect errors	$F(1,99) = 1.2$	$\uparrow ns$		
Arm omission errors	$F(1,99) = 0.4$	$\uparrow ns$		
Aborted choices	$F(1,99) = 4.6$	$\uparrow p < 0.0068$	0.065	Sig.
% Preferred choice angle	$F(1,99) = 1.6$	$\downarrow ns$		
% Choice angle repetitions	$F(1,99) = 1.4$	$\downarrow ns$		
% Chaining	$F(1,99) = 1.2$	ns		
<i>Spontaneous alternation (Orsay: 11 KO, 15 Wt)</i>				
Total visits	$F(1,24) = 5.4$	$\uparrow p < 0.0291$	0.144	Sig.
% All different in four choices	$F(1,24) = 1.4$	$\downarrow ns$		
% Different in four choices	$F(1,24) = 3.0$	$\downarrow p < 0.0949$	0.100	

Note: effects shown in same way as in Table 1

was challenged by giving only two trials a day (Fig. 4A, Table 3). In a probe trial given after 10 days of training Rsk-2 deficient mice searched normally according to % quadrant time, but their annulus crossing index was reduced and they spent more time in the wall zone (Fig. 4B, Table 3). However, in a second probe trial, 24 h after four additional days of training, they were no longer impaired. To challenge the mice with a more complex spatial problem, a further cohort of mice was tested on a serial reversal task. In a series of five spatial problems, each mouse was required to orient to a new goal position as soon as it had learned to escape reliably. Rsk2-deficient mice performed excellently in the preparatory cue navigation task (Fig. 4D), but needed more trials than controls to learn the five spatial problems (Fig. 4E). There was no significant effect of genotype on cumulative escape latency and swim path length, but the values of Rsk2-deficient mice were on average higher than in controls (Table 3). The impairment in this task did not become more severe with an increasing number of platform relocations. Rather, the data suggested that the impairment was most severe in the first task of the series (Fig. 4E). Also, there was no evidence of prolonged searching at the previous platform locations (Fig. 4F).

Discussion

Coffin Lowry syndrome is caused by mutations in the Rsk2 gene (Trivier et al. 1996) and has long been recognized as a rare but severe form of syndromic X-linked mental retardation. To date, little development has been made to aid the understanding of the nature and etiology of cognitive deficits associated with the illness. Here, we have conducted a battery of cognitive and non-cognitive behavioral tests and show, for the first time, that Rsk2-deficient mice display a

range of behavioral disturbances, including impairments in spatial learning and memory and alterations in exploratory behavior. Our main finding is that Rsk2-deficient mice show impaired acquisition of several place navigation tasks in the water-maze as well as reduced spatial reference memory after an interval of 9 days. By contrast, spatial reference memory after an interval of 24 h, as well as recognition memory for novel objects or the spatial configuration of objects were preserved in Rsk2-deficient mice. Further, a small impairment on the 8-arm radial maze suggests a mild deficit in spatial working memory.

The acquisition deficits in the water-maze were robust as they were observed under various spatial learning procedures conducted in the hands of two independent laboratories. The magnitude of the impairment clearly depended on the training schedule. It was minimal when six trials were given per day with an ITI of 45 min and became more severe when training trials were either massed with shorter ITIs or spread out across days. However, even the most severe deficit was only partial, allowing the mice to acquire the task with a delay after additional training. Together with the observation that the acquisition deficit was associated with increased wall hugging this suggests that the deficit may, at least in part, be due to a general difficulty in adapting to the test situation and to engage in the initial learning steps (Lipp and Wolfer 1998; Wolfer et al. 1998). The fact that the impairment of Rsk2-mice in the serial reversal procedure was marginal and did not increase across tasks with increasing spatial challenge supports this interpretation. The absence of perseverant searching at previous platform locations indicates that Rsk-2 deficient mice adapt well to changing spatial configurations within a given experimental setting.

As non-cognitive factors may interfere with performance in tests of learning and memory, we also

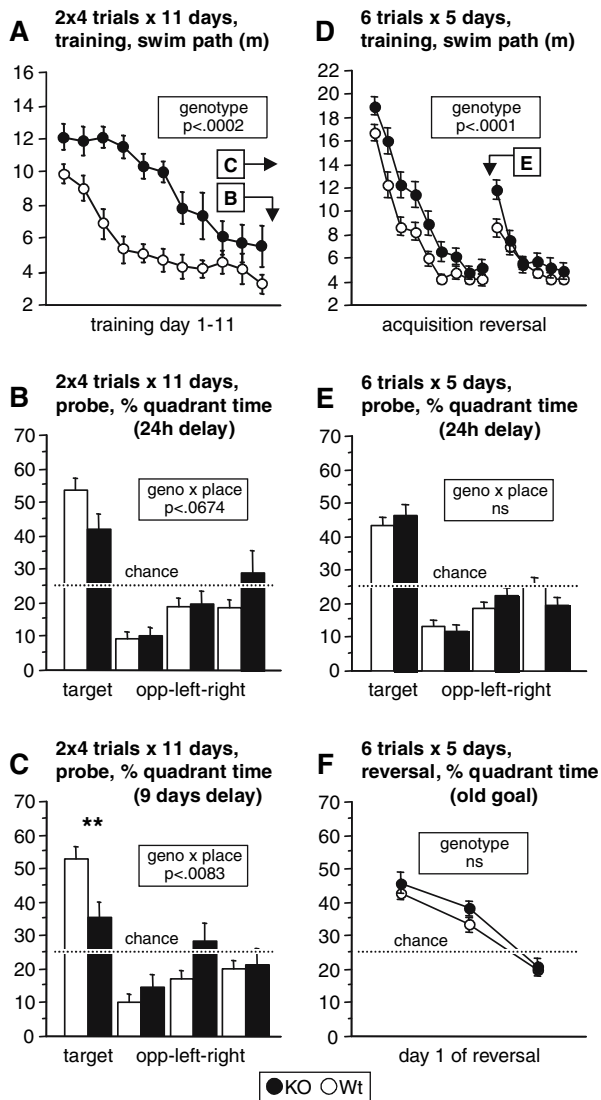


Fig. 3 Water-maze learning in place navigation procedures with two blocks of four trials per day (60 s ITI) for 11 days (Orsay: 10 KO, 15 Wt, A–C) and with six trials per day (45 min ITI) for 5 days (Zurich: 45 KO, 52 Wt, D–F). **(A)** Swim path to reach the hidden platform. Each point represents one training day. Rsk2-deficient mice showed poorer performance and delayed learning (genotype $F(1,23) = 19.1$, $p < 0.0002$, time $F(1,10) = 26.3$, $p < 0.0001$, genotype \times time $F(10, 230) = 3.614$, $p < 0.0002$). **(B)** Percent time spent in the target, opposite, adjacent left and adjacent right quadrants during the first probe trial 24 h after completion of training (place $F(3,69) = 31.1$, $p < 0.0001$, genotype \times place $F(3,69) = 2.5$, $p < 0.0674$; target versus 25% chance in Wt: $t(14) = 8.0$, $p < 0.0001$, KO $t(9) = 3.5$, $p < 0.0064$). **(C)** Percent time spent in the target, opposite, adjacent left and adjacent right quadrants during the second probe trial 9 days later (place $F(3,69) = 20.4$, $p < 0.0001$, genotype \times place $F(3,69) = 4.2$, $p < 0.0083$; target versus 25% chance in Wt: $t(14) = 7.1$, $p < 0.0001$, KO $t(9) = 2.4$, $p < 0.0380$; target Wt versus KO $t(23) = 2.9$, $p < 0.0079$). **(D)** Swim path length during acquisition and reversal training. Each point represents two subsequent trials. Rsk2-deficient mice performed more poorly during most of acquisition and at the beginning of reversal (genotype $F(1,85) = 17.1$, $p < 0.0001$, time $F(14,1190) = 65.2$, $p < 0.0001$, genotype \times time $F(14,1190) = 1.8$, $p < 0.0295$). **(E)** Percent time spent in the target, opposite, adjacent left and adjacent right quadrants during the probe trial 24 h after completion of 3 days of acquisition training. Rsk2-deficient mice did not differ from controls with respect to their preference for the trained quadrant (place $F(3,255) = 37.5$, $p < 0.0001$, genotype \times place $F(3,255) = 0.9$ ns; target versus 25% chance: $t(96) = 9.2$, $p < 0.0001$). **(F)** Percent time spent in the previous goal quadrant during the first day of reversal training. Each point represents two subsequent trials. Rsk2-deficient mice abandoned searching in the previous target quadrant as rapidly as controls (genotype: $F(1,85) = 2.2$ ns, time $F(2,170) = 69.2$, $p < 0.0001$, genotype \times time $F(2,170) = 0.3$ ns)

evaluated the animals' motor abilities and emotional reactivity. Smaller body weight and size have been reported both in Rsk2-deficient mice (Dufresne et al. 2001; El-Haschimi et al. 2003; Yang et al. 2004) and children with CLS (Hanauer and Young 2002), and a different line of Rsk2-deficient mice has been shown to perform badly on a rotating rod (Dufresne et al. 2001). Such physical effects of the mutation could potentially interfere with learning and memory performance. Direct body length measurements were not taken in the present study, but body size as measured indirectly by the video-tracking system was normal. The reduction of body weight was modest in our relatively young Rsk2-deficient mice, which may be explained by the fact that the smaller weight of Rsk2-deficient mice is due to reduced accumulation of adipose tissue with age (El-Haschimi et al. 2003). Moreover, Rsk2-deficient mice used in the present study showed normal

performance in sensorimotor tests, though a marginal deficit in the traction reflex test suggests that a mild alteration of motor coordination cannot be completely ruled out. However, the data show that differences in swim speed in the water-maze or abnormal swimming patterns such as circling could not account for the deficit observed in spatial learning performance. Rsk2-deficient mice also showed normal locomotor activity during open-field exploration, indicating that the effects of the mutation on the musculoskeletal system did not interfere with performance in these tests. The only clear change in locomotion patterns was an increase in acceleration in the mutant mice during bouts of progressive locomotion in a large open-field. This phenomenon, also described as darting behavior, is a robust trait of some inbred mouse strains, and has been reliably used to differentiate DBA/2 from C57BL/6 mouse strains (Kafkafi et al. 2003b). It is not known whether these strain differences are due to central or peripheral mechanisms, therefore it is not clear whether the more pronounced darting in Rsk2-deficient mice results from changes in the musculo-

Table 3 Factorial ANOVA of water-maze learning

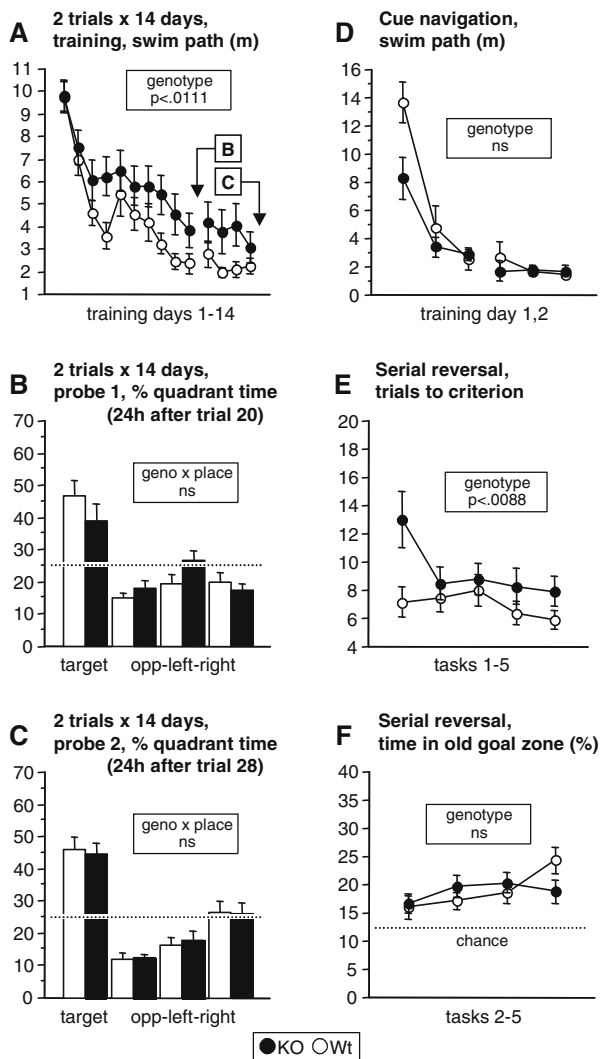
	<i>F</i> (df1,df2)	Type-I <i>p</i>	ω^2_p	FDR
<i>2 × 4 trials × 11 days (Orsay: 10 KO, 15 Wt)</i>				
Average escape latency	<i>F</i> (1,23) = 25.5	↑ <i>p</i> < 0.0001	0.495	Sig.
Average swim path length	<i>F</i> (1,23) = 19.1	↑ <i>p</i> < 0.0002	0.420	Sig.
% Time in wall zone	<i>F</i> (1,23) = 25.5	↑ <i>p</i> < 0.0001	0.495	Sig.
Average swim speed	<i>F</i> (1,23) = 6.2	↓ <i>p</i> < 0.0201	0.173	Sig.
Probe (24 h): annulus crossing index	<i>F</i> (1,23) = 2.6	↓ ns		
Probe (24 h): % time in wall zone	<i>F</i> (1,23) = 4.1	↑ <i>p</i> < 0.0555	0.109	
Probe (9 days): annulus crossing index	<i>F</i> (1,23) = 4.9	↓ <i>p</i> < 0.0364	0.136	Sig.
Probe (9 days): % time in wall zone	<i>F</i> (1,23) = 5.3	↑ <i>p</i> < 0.0306	0.147	Sig.
<i>6 trials × 5 days (Zurich: 45 KO, 52 Wt)</i>				
Average escape latency	<i>F</i> (1,85) = 11.6	↑ <i>p</i> < 0.0010	0.098	Sig.
Average swim path length	<i>F</i> (1,85) = 17.2	↑ <i>p</i> < 0.0001	0.143	Sig.
% Time in wall zone	<i>F</i> (1,85) = 2.4	↑ ns		
Number of wall approaches	<i>F</i> (1,85) = 7.3	↑ <i>p</i> < 0.0082	0.061	Sig.
Average swim speed	<i>F</i> (1,85) = 0.1	ns		
Time spent floating (log)	<i>F</i> (1,85) = 1.0	ns		
Circling index	<i>F</i> (1,85) = 4.0	ns		
Path efficiency	<i>F</i> (1,85) = 5.7	↓ <i>p</i> < 0.0192	0.046	Sig.
% Path parallel to border	<i>F</i> (1,85) = 13.9	↑ <i>p</i> < 0.0003	0.118	Sig.
Probe (trial 19): annulus crossing index	<i>F</i> (1,85) = 3.3	↑ <i>p</i> < 0.0713	0.026	
Probe (trial 19): % time in wall zone	<i>F</i> (1,85) = 4.6	↑ <i>p</i> < 0.0345	0.036	Sig.
<i>2 trials × 14 days (Zurich: 11 KO, 13 Wt)</i>				
Average escape latency	<i>F</i> (1,22) = 5.3	↑ <i>p</i> < 0.0318	0.151	Sig.
Average swim path length	<i>F</i> (1,22) = 7.8	↑ <i>p</i> < 0.0106	0.221	Sig.
% Time in wall zone	<i>F</i> (1,22) = 5.4	↑ <i>p</i> < 0.0296	0.155	Sig.
Number of wall approaches	<i>F</i> (1,22) = 13.2	↑ <i>p</i> < 0.0015	0.336	Sig.
Average swim speed	<i>F</i> (1,22) = 1.4	ns		
Time spent floating (log)	<i>F</i> (1,22) = 0.8	ns		
Circling index	<i>F</i> (1,22) = 0.2	ns		
Path efficiency	<i>F</i> (1,22) = 7.6	↓ <i>p</i> < 0.0114	0.216	Sig.
% Path parallel to border	<i>F</i> (1,22) = 14.2	↑ <i>p</i> < 0.0010	0.356	Sig.
Probe (trial 21): annulus crossing index	<i>F</i> (1,22) = 4.4	↓ <i>p</i> < 0.0480	0.124	Sig.
Probe (trial 21): % time in wall zone	<i>F</i> (1,22) = 4.7	↑ <i>p</i> < 0.0416	0.133	Sig.
Probe (trial 30): annulus crossing index	<i>F</i> (1,22) = 0.3	ns		
Probe (trial 30): % time in wall zone	<i>F</i> (1,22) = 0.2	ns		
<i>Serial reversal (Zurich: 15 KO, 13 Wt)</i>				
Cumulative escape latency	<i>F</i> (1,26) = 5.1	↑ <i>p</i> < 0.0333	0.127	
Cumulative swim path length	<i>F</i> (1,26) = 6.1	↑ <i>p</i> < 0.0203	0.155	
Average time in wall zone	<i>F</i> (1,26) = 0.7	ns		
Cumulative number of wall approaches	<i>F</i> (1,26) = 5.5	↑ <i>p</i> < 0.0267	0.140	
Average swim speed	<i>F</i> (1,26) = 0.9	ns		
Cumulative time spent floating (log)	<i>F</i> (1,26) = 0.8	ns		
Average circling index	<i>F</i> (1,26) = 1.0	ns		
Average path efficiency	<i>F</i> (1,26) = 1.5	↓ ns		
Average % path parallel to border	<i>F</i> (1,26) = 1.6	↑ ns		

Note: effects shown in same way as in Table 1.

skeletal system or is due to a central effect of the mutation.

In the spatial navigation tasks, Rsk-2 mutant mice showed increased wall hugging behavior. Their tendency to orient toward the wall varied across protocols and correlated well with the magnitude of the performance deficit (Table 3). It has been proposed that wall-hugging may result from increased anxiety in the water maze (Hodges 1996; Wolfer et al. 1998). However, we found that these mice showed no indication of increased anxiety-related responses in the open-field, the elevated plus maze or the O-maze. Instead, they tended to show hyper-responsiveness toward changes

in the environment. Rsk2-deficient mice made more head dips on the O-maze, showed increased exploration of the arena during the emergence test, visited the arms more frequently during the spontaneous alternation task, and reacted more strongly when new objects were added to a familiar arena during the first training day of the object exploration and recognition tasks. In general, our battery of non-cognitive tests suggest that Rsk2-deficient mice do not suffer from increased anxiety and are not generally hyperactive but tend to be hyper-responsive to environmental stimuli. Therefore, we believe that the thigmotaxic behavior in the water-maze is not indicative of an anxiety-induced



◀ **Fig. 4** Water-maze learning in a place navigation procedure of two trials per day during 14 days with 30 s ITI (Zurich, 11 KO, 13 WT, A–C) and in a cue navigation procedure that was followed by a serial reversal task (Zurich, 15 KO, 13 Wt; D–F). **(A)** Swim path length while learning to find the hidden platform. Each point represents the average of the two trials run on the same day. Swim paths of Rsk2-deficient mice were longer than those of controls (genotype $F(1,22) = 7.7$, $p < 0.0111$, time $F(13,286) = 18.8$, $p < 0.0001$, genotype \times time $F(13,286) = 0.6$ ns). **(B)** Percent time spent in the target, opposite, adjacent left and adjacent right quadrants during the first probe trial 24 h after 10 days of training in the 2 trials \times 14 days procedure. The mice spent more time than expected by chance in the target quadrant and there was no significant effect of genotype on the preference for the target quadrant (quadrant $F(3,22) = 19.2$, $p < 0.0001$, genotype \times place, $F(3,22) = 1.4$ ns, time in target quadrant versus 25% $t(23) = 5.0$, $p < 0.0001$). **(C)** Percent time spent in the target, opposite, adjacent left and adjacent right quadrants during a second probe trial 24 h after further 4 days of training in the 2 trials \times 14 days procedure. Again, the mice spent more time than expected by chance in the target quadrant and there was no significant effect of genotype on the preference for the target quadrant (quadrant $F(3,22) = 38.0$, $p < 0.0001$, genotype \times place $F(3,22) = 0.1$ ns, time in target quadrant versus 25% $t(23) = 8.3$, $p < 0.0001$). **(D)** Swim path length during training in the cue navigation task. Each point represents two successive trials. Rsk2-deficient mice were not impaired in this test. During the first two trials they were even more efficient than controls in reaching the platform (genotype $F(1,26) = 1.8$ ns, time $F(5,130) = 47.1$, $p < 0.0001$, genotype \times time $F(5,130) = 4.4$, $p < 0.0010$; time in Wt $F(5,60) = 33.2$, $p < 0.0001$, in KO $F(5,70) = 14.0$, $p < 0.0001$). **(E)** Trials needed to reach criterion in tasks 1–5 of the serial reversal procedure. Rsk2-deficient mice needed more training trials to reach criterion, especially during the first task (genotype $F(1,26) = 8.0$, $p < 0.0088$, task $F(4,104) = 2.1$, $p < 0.0895$, genotype \times task $F(4,104) = 1.5$ ns). **(F)** Average % time spent in the target zone of the previous task during tasks 2–5 (chance 12.5%). Irrespective of genotype the mice spent more time in the previous goal zone than expected by chance (genotype $F(1,26) = 0.1$ ns, task $F(3,78) = 2.7$, $p < 0.0516$, genotype \times task $F(3,78) = 1.9$ ns)

inability to learn the spatial memory task, but may well reflect a general maladaptive reaction to the unfamiliar test situation (Lipp and Wolfer 1998; Wolfer et al. 1998).

Taken together, Rsk2-deficient mice displayed selective learning impairments, to which impaired adaptation to the test environment likely makes a strong contribution, along with a moderate hyper-reactivity to environmental change. How does this compare to the behavioral changes seen in other models of human mental retardation? Table 4 gives a synopsis of findings reported in some other mouse models of monogenic forms of syndromic and non-syndromic mental retardation. It is evident that behavioral changes differ between different mouse models and the cognitive alterations seem less severe in mice than in mentally retarded humans. However, it should be kept in mind that the definition of mental retardation implies significant limitations both in

intellectual functioning (IQ < 70) and in adaptive behavior, but not complete loss of cognitive abilities. The sole use of IQ measures does not provide an adequate, or sufficient portrayal of the patients' entire repertoire of cognitive and intellectual functioning, as global IQ scores may mask the complex profile of the cognitive deficits and understate their wide-ranging effect on intellectual and social functions (Aylward 2002; Schalock et al. 1994). In CLS patients there is a marked variability in the severity of cognitive deficits, even between siblings, and environmental or other non-cognitive factors can determine the severity of mental retardation and how CLS patients cope with cognitive demands. Although it is debatable whether all features of human mental retardation can be modeled in rodents, several mouse models of mental retardation have been characterized by some mild,

Table 4 Behavioral changes in mouse models of monogenic syndromic and non-syndromic human mental retardation and selected references

Gene, human disease, mouse model	Exploratory behavior, activity	Learning and memory	Other observations
<i>FMR1</i> (FMRP, fragile X mental retardation protein, RNA binding protein that modulates translation), Fragile X syndrome (MR, macroorchidism, facial dysmorphisms), FMR1-KO (Bakker et al. 1994)	Increased exploratory behavior, reduced anxiety-related responses (Bakker et al. 1994; Mineur et al. 2002; Peier et al. 2000)	Normal fear conditioning (Peier et al. 2000), moderate impairment on radial-maze (Mineur et al. 2002), reversal learning in water-maze mildly impaired (Bakker et al. 1994)	Increased startle response (Nielsen et al. 2002), enhanced prepulse inhibition, audiogenic seizures (Chen and Toth 2001), impaired rotarod learning (Peier et al. 2000), reduced social interaction (Mineur et al. 2006)
<i>FMR2</i> (FMR2P, putative transcription factor), FRAXE syndrome (variable X-linked MR), FMR2-KO (Gu et al. 2002)	Normal open-field exploration, normal in light/dark box (Gu et al. 2002)	Impaired contextual fear conditioning, normal tone fear conditioning (Gu et al. 2002)	Normal startle response and prepulse inhibition, normal on rotarod (Gu et al. 2002)
<i>FRX2</i> (autosomal FMR1 homolog), FRX2-KO (Bontekoe et al. 2002)	Hyperactive in open-field (Bontekoe et al. 2002)	Impaired contextual fear conditioning, delayed place navigation acquisition (Bontekoe et al. 2002)	Impaired on rotarod, reduced prepulse inhibition, reduced pain sensitivity (Bontekoe et al. 2002)
<i>GDI1</i> (regulator of Rab family small GTPases), non-syndromic X-linked severe MR, GDI1-KO (D'Adamo et al. 2002; Ishizaki et al. 2000)	Open-field, O-maze, light/dark-box normal (D'Adamo et al. 2002)	Marginal water-maze place navigation deficit, normal delayed cue fear conditioning, impaired trace fear conditioning, impaired radial maze learning (D'Adamo et al. 2002)	Reduced aggression (D'Adamo et al. 2002)
<i>MECP2</i> (methyl-CpG-binding protein 2), Rett syndrome (X-linked MR, neurological deficits), conditional MECP2-KO (Guy et al. 2001)		Impaired water-maze place navigation, contextual fear conditioning, and social recognition (Moretti et al. 2006)	Motor deficits, death at 6–12 weeks, later onset of symptoms in heterozygous mice (Guy et al. 2001)
<i>L1</i> (Ig superfamily cell adhesion protein), CRASH syndrome (X linked MR, spastic paraplegia, hydrocephalus, corpus callosum hypoplasia), L1-KO (Cohen et al. 1998; Dahme et al. 1997)	Stereotyped circling in open-field (Fransen et al. 1998)	Slightly impaired water-maze place navigation, normal passive avoidance (Fransen et al. 1998)	Reduced startle response, reduced prepulse inhibition (Irintchev et al. 2004), reduced pain sensitivity (Dahme et al. 1997)
<i>ARHGEF6</i> (guanine nucleotide exchange factor for Rho GTPases), non-syndromic X-linked severe MR (Kutsche et al. 2000), ARHGEF6-KO	Disinhibited object exploration (Wolfer et al. 2005)	Navigation errors and perseverance in the water-maze place navigation task (Wolfer et al. 2005)	
<i>PAH</i> (phenylalanine hydroxylase), phenylketonuria (autosomal recessive MR, neurological deficits, epilepsy), Pah(enu2) mouse (McDonald et al. 1990)		Impaired olfactory discrimination, impaired latent learning (Zagreda et al. 1999), impaired spatial and non-spatial recognition (Cabib et al. 2003)	
<i>NF1</i> (neurofibromin, negative regulator of Ras), neurofibromatosis type 1 (autosomal dominant, mild MR, tumors), NF1+/- mouse (Silva et al. 1997)	Normal open-field exploration (Silva et al. 1997)	Normal cued fear conditioning, mild place-navigation deficit corrected by overtraining (Silva et al. 1997)	

often transitory, learning deficits, that may be combined with increased or stereotyped activity in exploration tests. The behavioral profile of Rsk2-deficient

mice conforms to this general pattern. Our own results indicate that maladaptive and exaggerated responses to environmental change may play a significant role in

the cognitive deficits found in *Rsk2*-deficient mice, suggesting that this should be further considered as a putative factor involved in the expression of mental retardation in rodents.

A possible route through which the loss of *Rsk2* gene function may contribute to the cognitive deficits observed in CLS patients and mutant mice is by affecting the function of the MAPK/ERK signaling pathway. This pathway plays a critical role in conveying signals generated at the receptor to the nucleus to trigger activity-dependent gene regulation that is considered to be an important mechanism for the maintenance of synaptic plasticity and the consolidation of long-term memories. A number of studies have shown that inhibition of ERK, the kinase upstream of RSK leads to rapidly decaying LTP (Davis 2000; English and Sweatt 1997) and an inability to form a number of different types of long-term memory, including fear associated memories (Atkins et al. 1998; Schafe et al. 2000), recognition memory (Kelly et al. 2003) and spatial memories (Blum et al. 1999; Selcher et al. 1999). One downstream target of RSK is the transcription factor CREB (Xing et al. 1996). In *aplysia* (Bartsch et al. 1995) and *drosophila* (Yin et al. 1994), CREB activation is necessary for consolidating memory and stabilizing synaptic plasticity. In several studies, this has also been claimed for rodents (Bourtchuladze et al. 1994; Bozon et al. 2003; Kida et al. 2002), although the evidence is not unequivocal (Balschun et al. 2003). It is noteworthy that the severity of acquisition deficits of CREB deficient mutant mice in water-maze tasks depended on the training schedule in a way reminiscent of the pattern observed in this study in *Rsk2*-deficient mice (Gass et al. 1998; Kogan et al. 1997). In human CLS patients, there is evidence for a direct relationship between the magnitude of *Rsk2*-mediated CREB phosphorylation and intelligence level (Harum et al. 2001). This suggests that insufficient *Rsk2* mediated CREB activation plays a prominent role in cognitive dysfunction of CLS patients. An important feature of the role of ERK and CREB in memory consolidation is that the cognitive impairment induced by inactivation of ERK and CREB is much more severe than that which we observed in *Rsk2*-deficient mice. Although *Rsk2* serves as an intermediate between ERK and CREB, the mitogen- and stress-activated kinase (MSK) is also activated by ERK and targets CREB. Therefore, *Rsk2* activation may not be the only means of activating CREB and there is growing evidence to suggest MSK may play a critical role in CREB-mediated transcription (Darragh et al. 2005; Wiggan et al. 2002). *Rsk2* can also phosphorylate histones (Sassone-Corsi et al. 1999)

and various transcription factors such as ATF4 (Yang et al. 2004), which have both been implicated in synaptic plasticity and memory formation (Alarcon et al. 2004; Chen et al. 2003; Costa-Mattioli et al. 2005; Korzus et al. 2004; Levenson et al. 2004). At present, however, the signaling mechanisms involved in mediating the cognitive disabilities observed here are not known.

In conclusion, the present results show that *Rsk2* gene mutation in mice results in a selective range of cognitive and behavioral alterations that is in keeping with the profile of alterations seen in other mouse models of human mental retardation. The specific deficits observed with these mice are also strengthened by the fact that it could be reproduced in two independent laboratories (Crabbe et al. 1999; Lewejohann et al. 2006; Wolfer et al. 2004). In all, the results strongly argue in favor of the *Rsk2*-deficient mouse as an animal model of mental retardation associated with the CLS syndrome. This should be useful to investigate neurobiological mechanisms responsible for cognitive dysfunction in CLS and to test therapeutic strategies to combat the effects associated with *Rsk2* gene mutations.

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